COMMONWEALTH of AUSTRALIA

PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT

We

SMITH KLINE - RIT
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BELGIUM

hereby apply for the grant of a Standard Patent for an invention entitled:

"ALPHA-1-ANTIPROTEASE PURIFICATION"

which is described in the accompanying complete specification.

Details of basic application(s):

<table>
<thead>
<tr>
<th>Number</th>
<th>Convention Country</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>836,868</td>
<td>UNITED STATES OF AMERICA</td>
<td>7th March 1986</td>
</tr>
</tbody>
</table>

The address for service is care of DAVIES & COLLISON, Patent Attorneys, of 1 Little Collins Street, Melbourne, in the State of Victoria, Commonwealth of Australia.

Dated this 5th day of March 1987

To: THE COMMISSIONER OF PATENTS

(a member of the firm of DAVIES & COLLISON for and on behalf of the Applicant).

Davies & Collison, Melbourne and Canberra.
In support of the Application made for a patent for an invention entitled: "ALPHA-1-ANTIPROTEASE PURIFICATION"

George Tasset,
of SMITH KLINE - RIT
of Rue du Tilleul, 13,
B-1320 Rixensart (Genval)
BELGIUM.

do solemnly and sincerely declare as follows:

1. (a) or (b) I am authorized by
SMITH KLINE - RIT
the applicant for the patent to make this declaration on its behalf.

2. (a) or (b) Alex Joseph BOLLEN and Marc HOYLAERTS
of Gaasbeekstraat, 65,
B-1711 Itterbeek,
BELGIUM.

Paul CHUCHANA
of "Les Cevennes",
Batiment 1.2
949, Avenue Professeur Louis Ravas
F-34100 Montpellier, FRANCE.

3. The basic application, as defined by Section 141 of the Act was made
in the UNITED STATES OF AMERICA
by ALEX JOSSEPH BOLLEN, PAUL CHUCHANA AND MARC HOYLAERTS
in the first application in respect of the invention the subject
of the application.

Declared at Rixensart this 23rd day of March, 1987.

G. TASET,
Authorized Official.
Alpha-1-antitrypsin (AAT) is an alpha-1-globulin present in serum and various other body fluids. As synthesized in the liver, mature AAT is a glycoprotein having a molecular weight of about 50,000 to 55,000 daltons.

Claim

1. A process for purifying AAT from an impure solution thereof which comprises:
   1) contacting the solution with an ion exchange adsorbent, and eluting the AAT therefrom;
   2) contacting the eluate from (1) with a thiol-disulfide exchange adsorbent and eluting the AAT therefrom;
   3) contacting the eluate from (2) with heparin bound to a solid carrier.
   4) contacting the effluent from (3) with a zinc-chelate adsorbent and eluting the AAT therefrom;
   5) contacting the eluate from (4) with an ion exchange adsorbent and eluting the AAT therefrom.
Complete Specification for the invention entitled:

"ALPHA-1-ANTIPROTEASE PURIFICATION"

The following statement is a full description of this invention, including the best method of performing it known to us:
TITLE

Alpha-l-Antiprotease Purification

FIELD OF THE INVENTION

This invention relates to purification of human alpha-l-antiprotease, herein referred to as alpha-l-antitrypsin.

BACKGROUND INFORMATION

Alpha-l-antitrypsin (AAT) is an alpha-l-globulin present in serum and various other body fluids. As synthesized in the liver, mature AAT is a glycoprotein having a molecular weight of about 50,000 to 55,000 daltons. Proteolytic enzymes inhibited by AAT include trypsin, chymotrypsin, pancreatic elastase, skin collagenase, renin and urokinase, as well as proteases of polymorphonuclear lymphocytes. Genetically acquired deficiency of AAT in humans is associated with various pathological conditions, especially emphysema and liver disease. See, for example, Morse, N. Eng. J. Med. 299 (19):1045-1048 (1978) and 299 (20):1099-1105 (1978); Tobin et al., Arch. Int. Med. 143 (7):1342-1348 (1982); and Carrell et al., Nature 298 (5872):329-334 (1982).

Elastase is a proteinase which breaks down lung tissue. Unchecked, its activity can result in emphysema. Gadek et al., Am. Rev. Respir. Dis. 127:S45 (1983) and Glaser et al., Am. Rev. Respir. Dis. 127:547-553 (1983), for example, have shown that AAT can be therapeutically useful in treatment of emphysema.

Because of its therapeutic utility and because comparatively large amounts are required for certain indications, such as replacement therapy for patients
genetically deficient in AAT, researchers have been looking for techniques to produce AAT in large quantities. Conventionally such techniques have involved purification of AAT from blood plasma.

Recent efforts have focused on production of AAT in transformed microorganisms or cells, especially E. coli and yeast, due to the promise of such techniques to produce large quantities of engineered gene products. Several researchers have reported success in such endeavors. However, a practical process for purifying such recombinant AAT useful in pharmaceutical applications is not available.

Laurell et al., J. Chromatog. 278:53-61 (1983) describe use of thiol-disulfide exchange chromatography to isolate AAT.

SUMMARY OF THE INVENTION

The invention is a process for purifying AAT from an impure solution thereof which comprises:

1) contacting the solution with an ion exchange adsorbent, and eluting the AAT therefrom;

2) contacting the eluate from (1) with a thiol-disulfide exchange adsorbent and eluting the AAT therefrom;

3) contacting the eluate from (2) with heparin bound to a solid carrier;

4) contacting the effluent from (3) with a zinc-chelate adsorbent and eluting the AAT therefrom;
5) contacting the eluate from (4) with an ion exchange adsorbent and eluting the AAT therefrom.

**DETAILED DESCRIPTION OF THE INVENTION**

The process of the invention comprises a series of adsorption steps each of which, individually, is carried out in accordance with standard techniques of protein purification. While the individual steps are, in a general sense, standard protein purification techniques, particular steps in a particular sequence must be selected from the myriad possibilities of process steps and sequences to achieve a purification process which is effective and efficient.

The AAT purified by the process of the invention can be identical to serum-derived AAT. The invention can also be applied to variant AAT molecules, that is, AAT molecules varying from natural AAT in secondary or tertiary structure, as may be the case for certain recombinant DNA-derived AAT proteins. Variant AAT molecules differing in primary structure can also be employed, provided that ability of the AAT to form disulfide bonds in the thiol-disulfide exchange step is not significantly adversely affected. Useful variant AAT molecules include, for example, those disclosed by Courtney et al., EP-A-114,777, Courtney et al., *Nature* 313:149 (1985) and Bollen et al., *DNA* 2:255 (1983).

The steps in the process of the invention are preferably carried out as chromatography steps, that is, continuous flow through an adsorbent, rather than batch-wise. The adsorbents used in the process of the invention comprise a solid carrier matrix through which various AAT-containing solutions are passed, thereby contacting a ligand for adsorbing the AAT or one or more impurities. Known such solid carriers include glass, silica, alumina.
and zirconia as well as organic carriers such as agarose, cellulose, dextran, polyamide, polyacrylamide and vinyl copolymers of bifunctional acrylates with various hydroxylated monomers. Commercially available carriers include Affi-gel®, Sephadex®, HP resins such as HP-20, XAD resins, Sepharose® and others.

The process employs an impure solution of AAT, that is, a solution in which AAT is less than 25% pure and typically is less than 10-15% pure. Such solution can be, for example, serum, medium from a producing bacterial or other cell culture in which the AAT is secreted or an extract from a bacterial or yeast host in which the AAT is soluble. A crude bacterial or yeast extract is preferably partially purified such as by selective ammonium sulfate precipitation followed by resolubilization or selective precipitation of contaminating proteins such as by use of a polyalkylene glycol. Techniques for preparing serum containing AAT are well-known. Techniques for preparing bacterial or other cell extracts containing soluble protein or for solubilizing protein in such extracts are also well-known. For example, E. coli transformants are collected by centrifugation and lysed for 30 min at 0°C in one-tenth volume of TES buffer (50 mM Tris, pH 8, 5 mM EDTA and 25% sucrose) supplemented with 0.5 mg/ml of lysozyme and 10 mg/ml of DNAse. Triton X100 is then added to a final concentration of 0.1%. The suspension is incubated at 0°C for 15 min and then brought to 80 mM Mg²⁺ by addition of 1M MgCl₂. This suspension is incubated for 5 min at 25°C. A supernatant is collected following centrifugation at 15,000 rpm for 10 min. Ammonium sulfate is added to the supernatant and the solution is centrifuged for 10 min at 10,000 rpm. The AAT is detected in the 50-75% ammonium sulfate fraction. This precipitate is resolubilized in buffer (50 mM Tris, pH 3, 25 mM NaCl) and dialyzed against the same buffer.
The first step in the process of the invention is an ion exchange step carried out in the presence of a mild detergent to remove lipids and most nucleic acids. An ion exchange column, preferably a DEAE column, such as DEAE-agarose, is equilibrated to approximately neutral pH, preferably 6.5, with a phosphate buffer containing a small amount of the detergent. After flowing the impure solution of AAT through the column, the column is washed with the equilibration buffer. Then the AAT is eluted with a salt, preferably about 150-250 mM NaCl in the equilibration buffer. The salt eluate is concentrated such as by filtration or dialysis. Ethylene diaminetetracetate (EDTA) is added to a final concentration of about 20 mM and the pH is raised to above neutral, e.g., >pH 7 to about pH 10, preferably pH 8-9.

In the second step, the concentrated eluate from step (1) is contacted with a thiol-disulfide exchange adsorbent. Such procedure utilizing immobilized human kappa (K) light chains is described by Laurell et al., J. Chromatogr. 278:53-61 (1983). AAT is eluted from the K light chains bound to a solid carrier with a reducing agent, preferably 4-nitrophenyl-disulfide-3,3'-dicarbonic acid and/or beta-mercaptoethanol to disrupt the disulfide bonds. The eluate is concentrated, desalted and adjusted to pH 7-8.

The concentrated eluate from step (2) is then contacted with heparin bound to a solid carrier, such as a heparin-agarose column, at ionic strength to adsorb most residual lipoproteins. The flow-through, that is, the effluent from this adsorbent contains the AAT.

In the fourth step, the effluent from the heparin step is contacted with immobilized Zn^{2+} ions, such as a zinc-chelate column, preferably an agarose column, to selectively adsorb AAT. The AAT is eluted by lowering the pH to below 6, for example, 5.5, or by step-wise elution
with histidine. Following acid elution, the pH is quickly readjusted to about neutral, that is, pH 6.5-7.5. The eluate is then dialyzed to remove eluting agents.

In the fifth step, the AAT solution is contacted with a second ion exchange adsorbent, preferably an amino-hexyl agarose column. AAT is specifically eluted using a salt gradient (150-250 mM NaCl). Pure AAT eluant is desalted such as by dialysis and concentrated, preferably by lyophilization.

The AAT resulting from this procedure can, if desired, be subjected to further purification steps to remove trace contaminants such as by affinity chromatography employing, for example, immobilized anhydrochymotrypsin or immobilized antibodies to AAT or to protein contaminants.

EXAMPLES

The Examples which follow are illustrative, and not limited, of a preferred purification process of the invention.

Example 1.

Purification of Mature AAT from Yeast

Recombinant plasmids expressing mature human AAT under the control of the yeast arg3 promoter, as previously described by Cabezon et al., Proc. Natl. Acad. Sci. USA 81:6594-6598 (1984), were used to express AAT in peptidase-deficient S. cerevisiae strains 10S442 (leu2-3, leu2-112, pep4-3) and TCY1 (ura3, leu2 defective).

Preliminary Precipitation: After mechanical disruption of cell membranes of about 1.5 to 2 kg of yeast, in an ethanol-dry ice cooled Dyno-Mill at 3000 rpm 'pumping speed around 5 l/h), in the presence of 20 mM N-mercaptoethanol, 5 mM EDTA, 1 mM PMSF and 1 mM benzamide (total volume 2.0 to 2.5 l) in 50 mM Tris-HCl buffer, pH 8.0, the pH of the crude extract was adjusted to 6.5 with 1 N HCl.
under stirring. Solid polyethylene glycol 1000 was added to 7%. Upon two hours of incubation, this crude extract was centrifuged at 16,000 g for 5 hours. Precipitated yeast membranes (around 500 ml) were resuspended in 1 l of 20 mM phosphate buffer pH 6.5, containing 7% PEG 1000 under sonication and recentrifuged for at least 5 h. Both supernatants were pooled (about 3 l), Triton WR1339 was added to 0.1%, and the pH was adjusted to 6.5. Total ionic strength was measured and adjusted to 70 mM of NaCl equivalent units by dilution with distilled water.

DEAE-chromatography: The AAT (herein α₁-PI) containing extract, partially depleted in cellular organelles and lipoproteins, was then charged onto a preparative DEAE-Sepharose Fast Flow column (5 x 26 cm) equilibrated in 20 mM phosphate buffer pH 6.5 containing 0.1% Triton and 0.01% NaN₃, at a flow rate of 600 ml/h. The column was washed with the starting buffer until the absorbance (280 nm) of the effluent fell below 2.0 (approximately 10 l), upon which an α₁-PI containing protein fraction was eluted stepwise with 150 mM of NaCl, added to the starting buffer. Residual fixed compounds were stepwise eluted with 1 M NaCl and 0.5 M NaOH respectively, after which the column was reequilibrated with the starting buffer. Upon concentrating the α₁-PI fraction to about 400 ml, EDTA was added to 20 mM and the pH was raised to 3.5 with solid Tris-powder.

Thiol-exchange chromatography: The slightly enriched material obtained in the previous step was applied onto a resin, consisting of human immunoglobulin light chains, idotype K, insolubilized on sepharose 4B, following cyanogen bromide coupling. As previously indicated, human K-light chains can be isolated from urine of myeloma patients, subject to the Bence-Jones syndrome. Upon charging batches of concentrated urine onto DEAE-Sepharose columns, the K-chains were incubated with saturating
concentrations of bis-(4-nitrophenyl)disulfide-3,3'-
dicarboxylic acid (DTNB) in 1 M Tris-HCl buffer pH 8.5,
containing 100 mM EDTA for four days at room temperature.
This procedure effectively dissociated dimers, which
appear upon formation of a disulfide bond between
C-terminal cysteines present in the K-chains, and
introduced a protective group from thiol-functions. After
separation of the excess of DTNB and TNB by sephadex G-25
chromatography, the protected K-chains were linked to 500
ml of Sepharose 4B, activated with CNBr just prior to
use. In this way 0.4 moles of thiols were introduced per
ml of gel, as calculated from the absorbance (412 nm) of
TNB detached from the resin upon complete reduction of
disulfide bridges with S-mercaptoethanol (3-SH).

The a1-PI solution was passed through a column
of insolubilized K-chains (2.6 x 94 cm), equilibrated in
50 mM Tris HCl buffer containing 200 mM NaCl, 20 mM EDTA,
0.1% Triton and 0.01% NaN3, at a flow rate of 100 ml/h.
The flow-through was treated with 20 mM 3-SH to reduce
disulfide bridges involving TNB and soluble proteins, and
was dialysed against the chromatography buffer, to which
active charcoal was added to accelerate elimination of
TNB. This flow-through (not entirely depleted in a1-PI)
subsequently was repassed onto the column as described for
the first passage.

After each passage, the column was washed with
the starting buffer and protein fraction containing
a1-PI was specifically eluted with an excess of TNB (1
mg/ml of DTNB + 0.25 mg/ml of dithiothreitol in the
starting buffer), which reverses the equilibrium
\[ S - S - \text{TNB} + \text{Protein SH} \rightarrow S - S - P + \text{TNB-SH}, \]
and hence detaches some proteins, amongst which is a1-PI.

A total elution was subsequently realized through
complete reduction of disulfide bridges with 20 mM 3-SH
added to the starting buffer. Prior to use, the column
was reactivated by passing 1 mg/ml of DTNB in the starting
buffer.
The specifically eluted $\alpha_1$-PI containing fraction was reduced with 20 mM 3-SH and dialysed against 20 mM phosphate buffer pH 7.5, to which active charcoal was added.

Alternatively upon reduction, the eluate was concentrated in an Amicon DC2 concentrator to 150 ml and desalted by sephadex G-25 chromatography in a column (5 x 50 cm) equilibrated with 20 mM phosphate buffer pH 7.5.

Heparin-agarose-chromatography: the dialysate of chromatographically desalted $\alpha_1$-PI solution was applied onto an heparin-agarose column (2.6 x 36 cm) equilibrated in 20 mM phosphate buffer pH 7.5 and washed with the starting buffer at a flow rate of 70 ml/h. The flow-through of this step was pooled and subsequently used. Proteins bound to the column were eluted with 1 M NaCl and discarded. Every fifth cycle, a solution of 2 M NaSCN was passed to detach molecules sticking to the agarose by non-specific interactions.

Zn-chelate-chromatography: The flow-through of the preceding step was deposited either as a pool of collected fractions, either directly by connecting the outlet of the heparin-agarose column to the inlet of a column of chelating sepharose (2.6 x 19 cm) charged with Zn$^{2+}$-ions, and equilibrated with 20 mM phosphate buffer pH 7.5. After completely charging the column, it was washed with 25 mM phosphate buffer pH 6.5, containing 0.5 M NaCl.

Specific elution of principally $\alpha_1$-PI was obtained upon lowering the pH to 5.5 or by gradient elution with histidine (0-25 mM) in the wash buffer. It was followed by a non-selective elution with 50 mM EDTA in 0.2 M Tris-HCl buffer pH 8.0, upon which the column was reequilibrated with ZnCl$_2$. In case of acid elution, the pH was raised immediately to about 7 by 1 ml of 1 M Tris-HCl buffer pH 8.0, present in the tubes during collection of the eluate. The pooled eluate was subsequently dialysed against 25 mM phosphate buffer pH 6.5, 25 mM NaCl.
AH-Sepharose chromatography: The \( \alpha_1 \)-PI solution, still slightly contaminated, is charged onto an amino-hexyl agarose column (2.6 x 18 cm) (AH-Sepharose, Pharmacia, Stockholm, Sweden) used as an ion-exchanger and equilibrated in 25 mM phosphate buffer pH 6.5, 25 mM NaCl. Adsorbed proteins were eluted by a total volume of 600 ml of a linear gradient of NaCl (25 to 300 mM) in the starting buffer, at a flow rate of 50 ml/h. Pure \( \alpha_1 \)-PI containing fractions were pooled and dialysed against 5 mM phosphate buffer pH 7, 15 mM NaCl and lyophilised. Upon dissolving the purified \( \alpha_1 \)-PI in one-tenth of the initial volume before lyophilisation in distilled water, ten-fold concentrated samples were obtained, having an adequate ionic strength for preservation of biological activity.

**EXAMPLE 2.**

**Purification and Activity of Various AAT Molecules**

The following AAT molecules were purified substantially by the procedure described in Example 1:

1. AAT deleted in 5 N-terminal amino acids and expressed in *E. coli*;
2. mature AAT (having N-met in place of the N-glu and lacking the ala at position 8) expressed in *E. coli*;
3. mature AAT (having N-met in place of the N-glu) expressed in yeast;
4. AAT from human plasma.

*E. coli* transformants were lysed to prepare cell extracts from which AAT was precipitated with ammonium sulfate substantially as described in the specification, above.

AAT was derived from plasma by standard techniques.

The activity of the four AAT proteins purified by the method of the invention was compared to that of a standard AAT preparation derived from serum in a standard
trypsin inhibition assay. The assay measures the inhibitory capacity of $\alpha_1$-antitrypsin toward trypsin. It consists of a microtest using the chromogenic substrate S2444 (L-pyroglutamylglycyl-L-arginine, p-nitroanilide hydrochloride, Kabi Diagnostica, Stockholm, Sweden). The polystyrene microtest plates were incubated for 1 hr with 1% bovine serum albumin and washed extensively with distilled water. $\alpha_1$-antitrypsin at various concentrations was incubated with a fixed amount of trypsin for 20 min at 37°C in a 200-$\mu$l final reaction volume of 0.1 M Tris-HCl pH 8.2/0.15 M NaCl/0.01 M EDTA/0.5% polyethylene glycol, $M_r$ 6000. Samples were cooled slowly at room temperature and then exposed to the chromogenic substrate (20 $\mu$l of aqueous solution of 0.003 M S2444). After 5 min at room temperature, the reaction was stopped with 50 $\mu$l of 50% acetic acid. Absorbance was read at 405 nm in a micro-ELISA automatic reader (Dynatech AM120). Calibration curves were determined for both trypsin and $\alpha_1$-antitrypsin. Samples to be assayed were diluted serially in reaction buffer.

The results, which are reported in the following table, demonstrate that the process of the invention can be used to purify AAT from various sources, including variant AAT molecules, without adversely affecting the protease-inhibiting activity thereof. Samples 1, 2, 3 and 4 are as defined above.
Activity assay of purified AAT produced in microorganisms or derived from pooled human plasma

<table>
<thead>
<tr>
<th>AAT concentration (µg/ml)</th>
<th>Optical density (A410 nm) (inhibition of trypsin activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>standard</td>
</tr>
<tr>
<td>0</td>
<td>0.95(0)</td>
</tr>
<tr>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.78(11)</td>
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<tr>
<td>1</td>
<td>0.63(33)</td>
</tr>
<tr>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.32(66)</td>
</tr>
<tr>
<td>2.50</td>
<td>0.24(74)</td>
</tr>
<tr>
<td>3.50</td>
<td>0.17(82)</td>
</tr>
<tr>
<td>4.50</td>
<td>0.1(90)</td>
</tr>
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</table>

Inhibition of trypsin activity is shown in parentheses.

The above description and examples fully disclose the subject invention including the preferred embodiments thereof. The invention, however, is not limited to the precise construction described therein but, rather includes all modifications and variations thereof which are encompassed within the scope of the claims which follow.
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A process for purifying AAT from an impure solution thereof which comprises:

   1) contacting the solution with an ion exchange adsorbent, and eluting the AAT therefrom;

   2) contacting the eluate from (1) with a thiol-disulfide exchange adsorbent and eluting the AAT therefrom;

   3) contacting the eluate from (2) with heparin bound to a solid carrier;

   4) contacting the effluent from (3) with a zinc-chelate adsorbent and eluting the AAT therefrom;

   5) contacting the eluate from (4) with an ion exchange adsorbent and eluting the AAT therefrom.

2. The process of claim 1 in which each step is carried out in chromatographic fashion and which comprises, in step (1), contacting the solution with a DEAE ion-exchange column in the presence of a detergent, and eluting the AAT therefrom with 150-250 mM NaCl;

   in step (2), contacting the eluate from (1) with an immobilized human K light chain column and eluting the AAT therefrom with a reducing agent;

   in step (4), contacting the effluent from (3) with a Zn-chelate column and eluting the AAT therefrom by lowering the pH to less than 6 or by contacting the bound AAT with histidine and immediately adjusting the pH to 6.5-7.5; and,

   in step (5), contacting the eluate from (3) with an amino-hexyl ion exchange column and eluting the AAT therefrom with NaCl (150 mM to 250 mM).
3. The process of Claim 1, substantially as hereinbefore described with reference to the Examples.

4. The steps, features, compositions and compounds referred to or indicated in the specification and/or claims of this application, individually or collectively, and any and all combinations of any two or more of said steps or features.

Dated this 5th day of March 1987

SMITH KLINE - RIT

By its Patent Attorneys

DAVIES AND COLLISON